

WHAT IS CLAIMED IS:

5        1. A tandem fluorescent protein construct comprising a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a linker moiety that couples the donor and acceptor moieties, wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer when the donor moiety is excited.

10      2. The construct of claim 1 wherein the donor moiety and acceptor moiety are Aequorea-related fluorescent protein moieties.

15      3. The construct of claim 2 wherein the donor moiety is P4-3 or W7 and the acceptor moiety is S65C or S65T.

20      4. The construct of claim 1 wherein the linker moiety comprises a cleavage recognition site for an enzyme.

25      5. The construct of claim 4 wherein the linker moiety is a peptide moiety.

20      6. The construct of claim 5 comprising a fusion protein including the donor moiety, the peptide moiety and the acceptor moiety in a single polypeptide.

25      7. The construct of claim 6 wherein the linker moiety comprises between about 5 amino acids and about 50 amino acids.

25      8. The construct of claim 7 wherein the linker moiety comprises between about 10 amino acids and about 30 amino acids.

25      9. The construct of claim 8 wherein the donor moiety is P4-3 or W7 and the acceptor moiety is S65C or S65T.

30      10. The construct of claim 7 comprising a cleavage recognition site for trypsin, enterokinase, HIV-1 protease, prohormone convertase, interleukin-1 $\beta$ -converting enzyme, adenovirus endopeptidase, cytomegalovirus assemblin, leishmanolysin,  $\beta$ -Secretase for APP, thrombin, renin, angiotensin-converting enzyme, cathepsin D or a kininogenase.

11. The construct of claim 6 wherein the donor moiety is positioned at the amino terminus of the polypeptide relative to the acceptor moiety.

5 12. The construct of claim 7 wherein the linker moiety comprises a cleavage site having a randomized amino acid sequence.

10 13. The construct of claim 1 wherein the linker moiety has a length between about 1 nm and about 10 nm.

15 14. The construct of claim 4 comprising a cleavage recognition site for  $\beta$ -lactamase.

20 15. The construct of claim 1 wherein the linker moiety comprises a cross-linker moiety.

25 16. A recombinant nucleic acid coding for expression of a tandem fluorescent protein construct, the construct comprising a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a peptide linker moiety in a single polypeptide, wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer when the donor moiety is excited.

30 17. The recombinant nucleic acid of claim 16 wherein the peptide linker moiety comprises a cleavage recognition site for a protease.

25 18. The recombinant nucleic acid of claim 17 wherein the donor moiety is selected from the group comprising W1B, Topaz, P4-3, and W7 and the acceptor moiety is selected from the group comprising Topaz, Emerald, S65C and S65T.

30 19. An expression vector comprising expression control sequences operatively linked to a sequence coding for the expression of a tandem fluorescent protein construct, the construct comprising a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a peptide linker moiety in a single peptide, wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer when the donor moiety is excited.

5           20. An expression vector of claim 19 adapted for function in a prokaryotic cell.

10          21. An expression vector of claim 19 adapted for function in a eukaryotic cell.

15          22. A host cell transfected with an expression vector comprising an expression control sequence operatively linked to a sequence coding for the expression of a tandem fluorescent protein construct, the construct comprising a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a peptide linker moiety in a single polypeptide, wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer when the donor moiety is excited.

20          23. The cell of claim 22 further comprising a protease that is not normally expressed by said cell.

25          24. The cell of claim 22 that is *E. coli*.

30          25. The cell of claim 22 that is a eukaryotic cell.

35          26. The cell of claim 22 that is a cultured mammalian cell.

40          27. A method for determining whether a sample contains an enzyme comprising:

45              contacting the sample with a tandem fluorescent protein construct which comprises a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a linker moiety that couples the donor and acceptor moieties and that comprises a cleavage recognition site specific for the enzyme, wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer when the donor moiety is excited;

50              exciting the donor moiety; and

55              determining the degree of fluorescence resonance energy transfer in the sample, whereby a degree of fluorescence resonance energy transfer that is lower than an expected amount indicates the presence of an enzyme.

60          28. The method of claim 27 for determining the amount of an enzyme in a sample wherein determining the degree of

fluorescence resonance energy transfer in the sample comprises determining the degree at a first and second time after contacting the sample with a tandem fluorescent protein construct, and determining the difference in the degree of fluorescence resonance energy transfer, whereby the difference in the degree of fluorescence resonance energy transfer reflects the amount of enzyme in the sample.

29. The method of claim 27 wherein the step of determining the degree of fluorescence resonance energy transfer in the sample comprises determining the amount of fluorescence from the donor moiety.

30. The method of claim 27 wherein the step of determining the degree of fluorescence resonance energy transfer in the sample comprises determining the amount of fluorescence from the acceptor donor moiety.

31. The method of claim 27 wherein the step of determining the degree of fluorescence resonance energy transfer in the sample comprises determining the ratio of the amount of fluorescence from the donor moiety and the amount of fluorescence from the acceptor moiety.

32. The method of claim 27 wherein the step of determining the degree of fluorescence resonance energy transfer in the sample comprises determining the excitation state lifetime of the donor moiety.

33. The method of claim 27 wherein the enzyme is a protease and the linker moiety is a peptide moiety having the cleavage recognition site.

34. The method of claim 33 wherein the donor fluorescent protein moiety is an Aequorea-related fluorescent protein.

35. The method of claim 34 wherein the donor moiety is P4-3 or W7 and the acceptor moiety is S65C or S65T.

36. A method of determining the amount of activity of an enzyme in a cell comprising the steps of:

providing a cell that expresses a tandem fluorescent protein construct, the construct comprising a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a peptide linker moiety, wherein the peptide linker moiety comprises a cleavage recognition amino acid sequence specific for the enzyme, and wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer when the donor moiety is excited;

exciting the donor moiety; and

determining the degree of fluorescence resonance energy transfer in the cell, whereby the degree of fluorescence resonance energy transfer relates the amount of enzyme activity in the cell.

37. The method of claim 36 wherein the cell is transfected with an expression vector comprising expression control sequences operably linked to a nucleic acid sequence coding for the expression of the enzyme.

38. The method of claim 37 wherein the donor fluorescent protein moiety is an Aequorea-related fluorescent protein.

39. The method of claim 38 wherein the donor moiety is P4-3 or W7 and the acceptor moiety is S65C or S65T.

40. The method of claim 36 wherein the step of providing a cell comprises inducing expression of the construct to produce a sudden increase in the expression of the construct, and the step of determining the degree of fluorescence resonance energy transfer comprises determining the degree at a first and a second time after expression of the construct and determining the difference between the first and second time, whereby the difference reflects the amount of enzyme.

41. A method of determining the amount of activity of an enzyme in a sample from an organism comprising the steps of:

providing a sample from an organism having a cell that expresses a tandem fluorescent protein construct, the

construct comprising a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a peptide linker moiety, wherein the peptide linker moiety comprises a cleavage recognition amino acid sequence specific for the enzyme, and wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer when the donor moiety is excited;

exciting the donor moiety; and

determining the degree of fluorescence resonance energy transfer in the sample, whereby the degree of fluorescence resonance energy transfer reflects the amount of enzyme activity in the cell.

42. A method for determining whether a compound alters the activity of an enzyme comprising the steps of:

contacting a sample containing a known amount of the enzyme with the compound and with a tandem fluorescent protein construct which comprises a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a linker moiety that couples the donor and acceptor moieties and that comprises a cleavage recognition site specific for the enzyme, wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer when the donor moiety is excited;

exciting the donor moiety; and

determining the amount of enzyme activity in the sample as a function of the degree of fluorescence resonance energy transfer in the sample.

43. The method of claim 42 wherein the enzyme is a protease and the compound is at a predetermined concentration of at least 1uM.

44. The method of claim 42 further comprising the step of comparing the amount of activity in the sample with a standard activity for the same amount of the enzyme, whereby a difference between the amount of enzyme activity in the sample and the standard activity indicates that the compound alters the activity of the enzyme.

45. A method for determining whether a compound  
alters the activity of an enzyme in a cell comprising the steps  
of:

5 providing first and second cells that express a  
tandem fluorescent protein construct, the construct comprising a  
donor fluorescent protein moiety, an acceptor fluorescent  
protein moiety and a peptide linker moiety, wherein the peptide  
linker moiety comprises a cleavage recognition amino acid  
sequence specific for the enzyme, and wherein the donor and  
10 acceptor moieties exhibit fluorescence resonance energy transfer  
when the donor moiety is excited;

15 contacting the first cell with an amount of the  
compound;

20 contacting the second cell with a different  
amount of the compound;

25 exciting the donor moiety in the first and second  
cell;

determining the degree of fluorescence resonance  
energy transfer in the first and second cells; and

30 comparing the degree of fluorescence resonance  
energy transfer in the first and second cells, whereby a  
difference in the degree of fluorescence resonance energy  
transfer indicates that the compound alters the activity of the  
enzyme.

46. A tandem fluorescent protein construct comprising  
a donor moiety, an acceptor moiety and a linker moiety that  
couples a donor and acceptor moiety, wherein one of the donor or  
acceptor moieties is a fluorescent protein and one is a  
non-protein compound fluorescent moiety, and wherein the donor  
and acceptor moieties exhibit fluorescence resonance energy  
transfer when the donor moiety is excited.

47. The construct of claim 46 wherein the fluorescent  
protein moiety is an Aequorea-related fluorescent protein moiety.

48. A method of testing for cleavage enzyme activity

comprising:

5           contacting a cleavage enzyme with a tandem fluorescent protein construct which comprises a donor fluorescent protein moiety, an acceptor fluorescent protein moiety and a linker moiety that couples the donor and acceptor moieties and that comprises at least one cleavage recognition site, wherein the donor and acceptor moieties exhibit fluorescence resonance energy transfer when the donor moiety is excited;

10           exciting the donor moiety; and

15           determining the presence of fluorescence resonance energy transfer between the donor and the acceptor moieties,

20           wherein a decrease in fluorescence resonance energy transfer indicates the presence of a cleavage recognition site that is cleaved by the cleavage enzyme.

25           49. The method of claim 48 wherein the cleavage enzyme is a protease.

30           50. The method of claim 49 wherein the cleavage enzyme is an orphan protease.

35           51. The method of claim 50 wherein the cleavage recognition site has a random amino acid sequence.

40           52. The method of claim 51 wherein the cleavage enzyme is contacted with the tandem fluorescent protein construct by expressing the cleavage enzyme and the tandem fluorescent protein construct in a cell.

45           52. The method of claim 51 wherein the cleavage enzyme is expressed using an inducible promoter and optionally exposing the cell to an inducer of the inducible promoter for less than two hours, wherein the cleavage enzyme is transiently expressed.

50           53. The method of claim 52 wherein the cleavage enzyme and the tandem fluorescent protein construct have a signal sequence directing expression of protein into a vesicle.

55           54. The method of claim 51 wherein the cell is part

of a library of individual clones, wherein different said clones have been transfected with tandem fluorescent protein constructs having different cleavage recognition sites.

5       55. The method of claim 54 further comprising selecting clones from said library that have cleavage recognition sites cleaved by proteases.

10      56. The method of claim 55 wherein the selecting of the clones comprises identifying the clones with a Fluorescent Activated Cell Sorter (FACS) or luminescent assay based sorter.

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